

Training module # WQ - 21

***Microbiological Laboratory
Techniques***

New Delhi, July 1999

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DHV Consultants BV & DELFT HYDRAULICS

with
HALCROW, TAHAL, CES, ORG & JPS

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1. Module context

This module introduces microbiological laboratory techniques to be used for analysis of coliforms bacteria can be used as indicators of pollution. Modules in which prior training is required to complete this module successfully and other related modules in this category are listed below.

While designing a training course, the relationship between this module and the others, would be maintained by keeping them close together in the syllabus and place them in a logical sequence. The actual selection of the topics and the depth of training would, of course, depend on the training needs of the participants, i.e. their knowledge level and skills performance upon the start of the course.

| No. | Module title | Code | Objectives |
|-----|--|---------|---|
| 1 | Basic water quality concepts ^a | WQ - 01 | <ul style="list-style-type: none">• Become familiar with common water quality parameters• Appreciate important water quality issues |
| 2 | Basic chemistry concepts ^a | WQ - 02 | <ul style="list-style-type: none">• Convert units from one to another• Understand the basic concepts of quantitative chemistry• Report analytical results with the correct number of significant digits |
| 3 | How to prepare standard solutions ^a | WQ - 04 | <ul style="list-style-type: none">• Recognise different types of glassware• Use an analytical balance and maintain it• Prepare standard solutions |
| 4 | Introduction to microbiology ^a | WQ - 20 | <ul style="list-style-type: none">• Classify different types of micro-organisms• Identify certain water borne diseases |
| 5 | Coliforms as indicators of faecal pollution | WQ - 22 | <ul style="list-style-type: none">• Identify the main water quality problems caused by micro-organisms• Explain why coliform bacteria are good indicators• Explain the principles of the coliform analysis method |
| 6 | How to measure coliforms | WQ - 23 | <ul style="list-style-type: none">• Measure total and faecal coliforms in water samples |

a - prerequisite

2. Module profile

| | | |
|---------------------------------------|---|--|
| Title | : | Microbiological Laboratory Techniques |
| Target group | : | HIS function(s): Q2, Q3, Q5, Q6 |
| Duration | : | 1 session of 60 min |
| Objectives | : | After the training the participants will be able to: <ul style="list-style-type: none">• Explain methods of bacteria identification• Discuss methods of bacteria enumeration• Follow methods of good laboratory practice |
| Key concepts | : | <ul style="list-style-type: none">• Culture of micro-organisms• Solid and liquid media• Laboratory techniques• Identification of micro-organisms |
| Training methods | : | Lecture, discussion exercises |
| Training tools required | : | Board, OHS, flipchart |
| Handouts | : | As provided in this module |
| Further reading and references | : | <ul style="list-style-type: none">• The Microbial World, Stanier et al, Prentice-Hall, 1986• Standard Methods: for the Examination of Water and Wastewater, APHA, AWWA, WEF/1995. APHA Publication |

3. Session plan

| No | Activities | Time | Tools |
|----|--|--------|------------|
| 1 | Preparations | | |
| 2 | Introduction: <ul style="list-style-type: none">• Introduce the subject of identifying bacteria in laboratories• Explain several general methods for identifying bacteria• Give advantages of the coliform indicator test | 10 min | OHS |
| 3 | Laboratory Procedures: <ul style="list-style-type: none">• Explain liquid and solid culture media• Illustrate how bacterial growth can be seen• Review sterilization procedures and aseptic methods | 15 min | OHS |
| 4 | Enumeration of Bacterial Populations <ul style="list-style-type: none">• Explain plate counts for solid media• Explain MPN estimation for liquid media | 15min | OHS |
| 5 | Sampling and Good Laboratory Practice <ul style="list-style-type: none">• Review sample collection procedures for bacteria• Explain good laboratory practice for microbiology• Stress importance of cleanliness | 10 min | OHS |
| 6 | Wrap up and Evaluation | 10 min | discussion |

4. Overhead/flipchart master

OHS format guidelines

| Type of text | Style | Setting |
|------------------------|-------------------------------|---|
| Headings: | OHS-Title | Arial 30-36, with bottom border line (not: underline) |
| Text: | OHS-lev1 OHS-lev2 | Arial 24-26, maximum two levels |
| Case: | | Sentence case. Avoid full text in UPPERCASE. |
| Italics: | | Use occasionally and in a consistent way |
| Listings: | OHS-lev1 OHS-lev1-Numbered | Big bullets. Numbers for definite series of steps. Avoid roman numbers and letters. |
| Colours: | | None, as these get lost in photocopying and some colours do not reproduce at all. |
| Formulas/ Equations | OHS-Equation | Use of a table will ease horizontal alignment over more lines (columns) Use equation editor for advanced formatting only |

Microbiological Laboratory techniques

- Culture media
- Aseptic techniques
- Identification
- Enumeration
- Good laboratory practices

Bacteriological quality of water

- A variety of procedures available to measure bacteriological quality of water:
 - *Total plate count at 20°C and at 37°C.*
 - *Presence of coliform bacteria as indicators of sewage contamination*
 - *Identification of specific pathogenic bacteria*
 - *Employing miscellaneous indicators and serological methods*

Bacteriological quality of water

- Identification of specific micro-organisms difficult & costly
- Simplified tests preferred to indicate sanitary quality of water:
 - *Tests for coliform group of bacteria (most common test)*
 - *total plate count (in some cases)*

Culture of micro-organisms

- Bacteria grown in laboratory under controlled conditions
 - *growth medium (solid or liquid) with nutrients and energy source*
 - *temperature*
 - *pH, salinity, oxygen*
 - *no competing organisms*
 - *no antibacterial substances*
- Typical solid growth media is 'agar', extracted from seaweed

Types of culture media

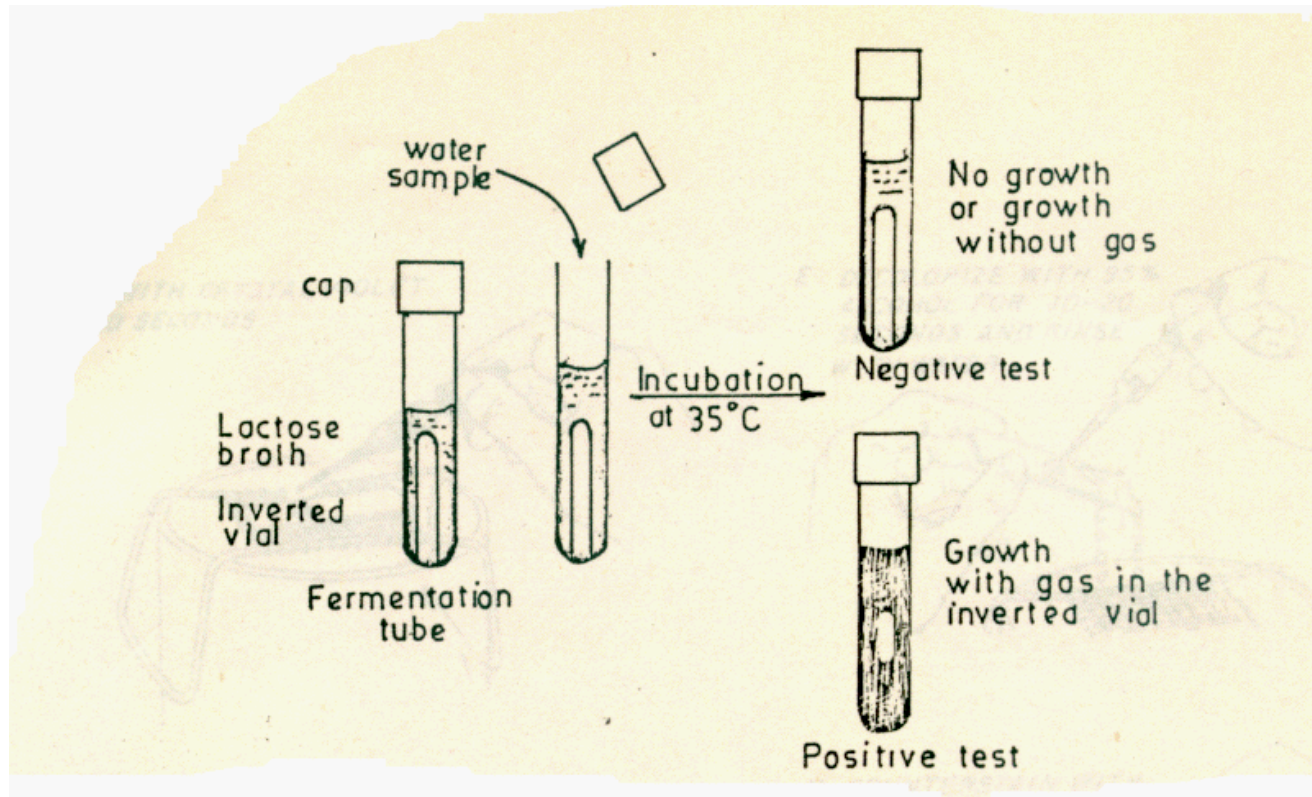
- General purpose
- Enrichment
- Selective

Culture in liquid medium

- In liquid media, bacterial growth demonstrated by:
 - *turbidity*
 - *change in colour of indicator (acid or alkali production)*
 - *gas production (breakdown of sugars, etc.)*
- Gas can be trapped in small inverted test tube:
(Durham tube)
- Gas production test used to indicate coliform bacteria

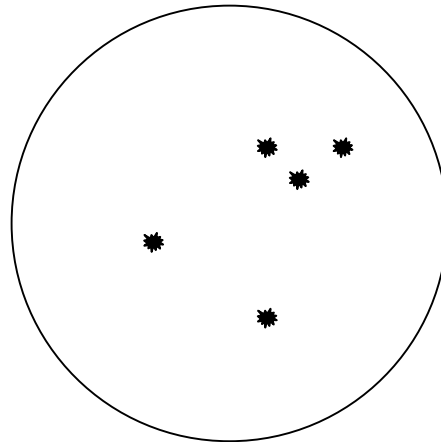
Culture in liquid medium

- Bacterial growth with gas production in liquid culture medium



Culture in solid medium

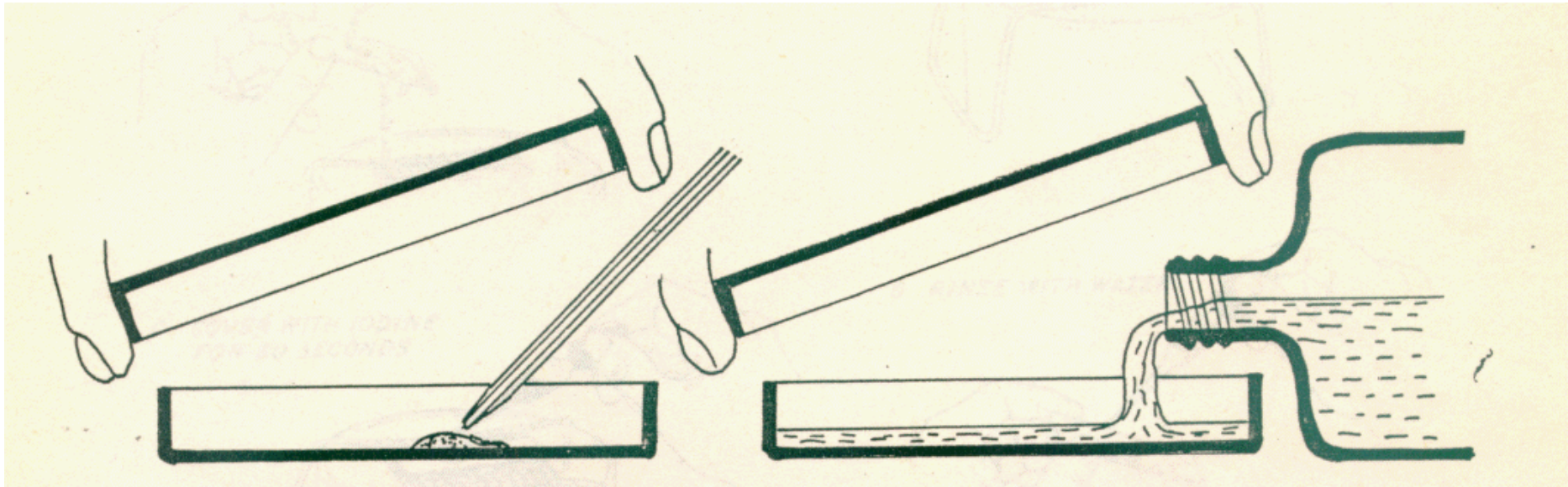
- On solid agar media, bacteria cannot move
- Cells grow locally, forming clusters or 'colonies'
- Colonies visible to the naked eye, can be counted



Petri dish with 5 bacterial colonies

Culture in solid medium

- Solid agar media often poured into shallow, covered (petri) dishes to harden
- Sample already in petri dish or added after hardening



Sterilisation

- Sterilise all equipment and materials:
 - *autoclave for steam sterilisation:*
 - *121°C for culture broths and dilution water, 15 minutes*
 - *oven for dry sterilisation:*
 - *170°C for pipettes in metal containers, 20-30 minutes*
- Aseptic laboratory techniques needed:
 - *no cross-contamination of samples and culture media with micro-organisms*

General aseptic techniques

- Wash hands
- Clean bench area with swab soaked in methylated spirit
- Don't touch any part of the container, pipette, etc., coming in contact with the sample or culture.
- Don't remove lid of a petri dish or cap of a test tube longer than absolutely necessary.

General aseptic techniques

- Lightly flame top of test tubes, ends of pipettes, necks and stoppers of bottles before and after adding/ withdrawing samples or inocula
- Transfer culture from one tube to a fresh tube (subculturing) using wire loop sterilised by heating to redness and cooled
- Always follow these methods to avoid contamination!

Enumerating bacterial populations

-Solid media: Plate count

*-Liquid media: MPN (Most Probably Number) estimation
less accurate than plate count*

Enumeration on solid media

- Plate count on solid media
 - *Individual colonies are counted, must be well separated*
 - *Each colony represents 1 cell*
- Dilution technique needed for high concentrations
 - *1 ml sample added to 9 ml 'diluent'*
 - *Diluent should be standard salt solution*
 - *Dilution series (5 or more) made*

Dilution technique for plate count

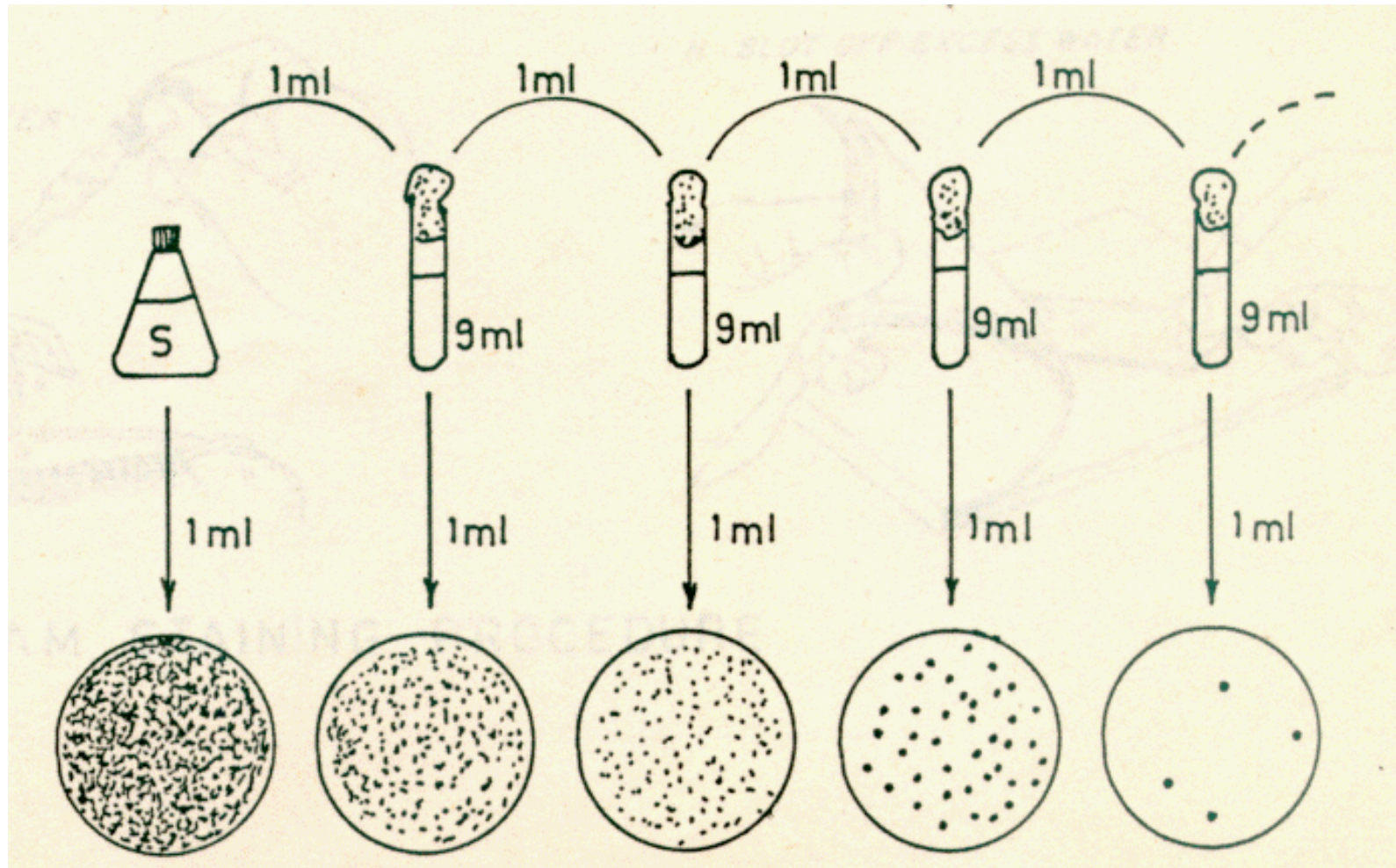


Plate count result

- Plate count, N, should be between 30 and 300
- Confidence limits (95%) calculated by:

$$\text{Upper limit} = N + 2 (2 + \sqrt{N})$$

$$\text{Lower limit} = N - 2 (1 + \sqrt{N})$$

- Count per ml must be multiplied by dilution factor

Enumeration in liquid media

- Used for coliform analysis
- Gas production by coliforms in selective medium
- Make a series of dilutions
- Inoculate sets of culture tubes with different dilutions
- Example with 3 dilutions:
 - *5 tubes with 10 ml sample*
 - *5 tubes with 1.0 ml sample*
 - *5 tubes with 0.1 ml sample*

Enumeration in liquid media

- Most Probable Number (MPN) of bacteria in sample based on number of tubes with gas production and volume of inoculum
- MPN /100 ml sample is a statistical estimate

Sampling procedures

- *samples must be representative of water body*
- *use sterilised bottles and follow aseptic procedures*
- *collect sample from 30 cm below surface (river, reservoir)*
- *leave some air space in bottle (2.5 cm)*
- *store samples at 4°C*
- ***ANALYSE SAMPLES WITHIN 8 HOURS OF COLLECTION***

Good laboratory practice for microbiology

- *important for health & safety of personnel (pathogens)*
- *sterilise all necessary equipment and media*
- *frequently disinfect hands and work surfaces*
- *keep flies, insects out of laboratory*
- *don't mouth pipette samples with (possible) high bacteria*
- *sterilise contaminated waste prior to disposal*

5. Evaluation sheets

6. Handout

Microbiological Laboratory techniques

- Culture media
- Aseptic techniques
- Identification
- Enumeration
- Good laboratory practices

Bacteriological quality of water

- A variety of procedures available to measure bacteriological quality of water:
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Culture of micro-organisms

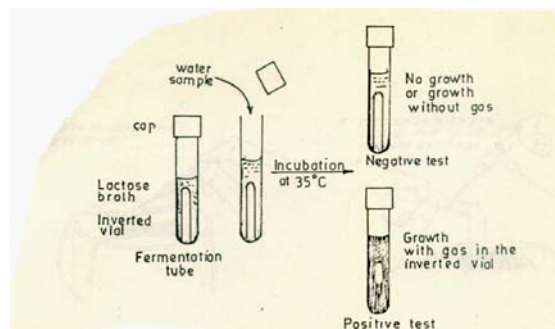
- Bacteria grown in laboratory under controlled conditions
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Types of culture media

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- Enrichment
- Selective

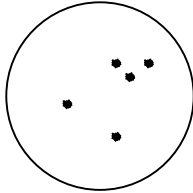
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- Bacterial growth with gas production in liquid culture medium



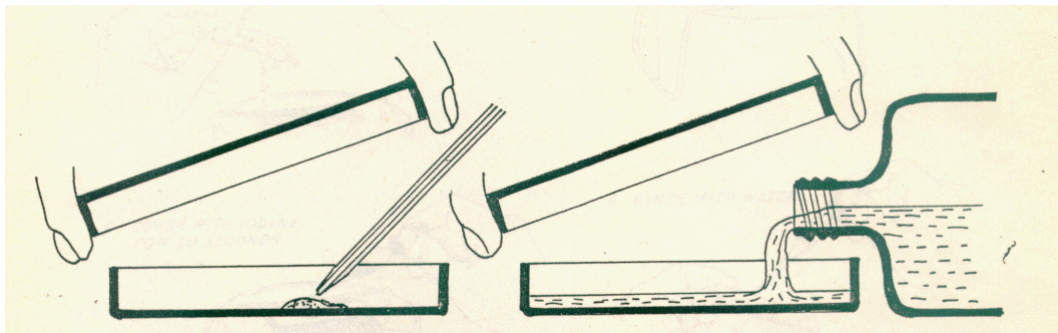
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General aseptic techniques

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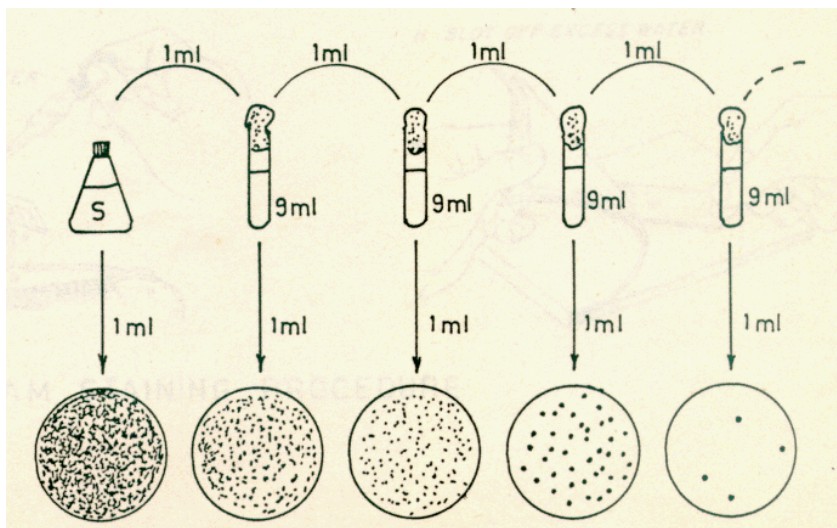


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Good laboratory practice for microbiology

- *important for health & safety of personnel (pathogens)*
- *sterilise all necessary equipment and media*
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- *don't mouth pipette samples with (possible) high bacteria*
- *sterilise contaminated waste prior to disposal*

Add copy of Main text in chapter 8, for all participants.

7. Additional handout

These handouts are distributed during delivery and contain test questions, answers to questions, special worksheets, optional information, and other matters you would not like to be seen in the regular handouts.

It is a good practice to pre-punch these additional handouts, so the participants can easily insert them in the main handout folder.

8. Main text

| | | Contents |
|-----------|---------------------------------------|-----------------|
| 1. | Introduction | 1 |
| 2. | Laboratory Procedures | 1 |
| 3. | Identification and enumeration | 2 |
| 4. | Sampling | 4 |
| 5. | Good Laboratory Practice | 4 |

Microbiological Laboratory Techniques

1. Introduction

Most bacteria in water are derived from contact with air, soil, living or decaying plants or animals, and faecal excrement. Many of these bacteria have no sanitary significance as they are not pathogenic and are not suspected of association with pathogenic micro-organisms. A variety of procedures has been used to measure the bacteriological quality of water:

1. Total plate count at 20°C and at 37°C.
2. Presence of coliform bacteria as indicators of sewage contamination
3. Identification of specific pathogenic bacteria
4. Employing miscellaneous indicators and serological methods

The specific identification of pathogenic bacteria requires large samples, a variety of laboratory procedures and is time consuming and costly. Such tests are therefore not applicable to routine testing and monitoring. Tests for coliform group of bacteria and in some cases the total plate count are considered adequate to indicate sanitary quality of water.

To aid identification the microbiologist normally uses techniques known as 'isolation' and 'cultivation'. Isolation is the separation of a particular organism from a mixed population whilst cultivation is the growth of that organism in an artificial environment (often in an incubator at a constant temperature because many micro-organisms only grow well within a limited range of temperatures).

2. Laboratory Procedures

Culture media

Bacteria are grown in laboratory by providing them with an environment suitable for their growth. The growth medium should contain all the correct nutrients and energy source and should be maintained at proper pH, salinity, oxygen tension and be free of antibacterial substances.

Such media can be either liquid or solid. A solid medium is generally the corresponding liquid medium made up with a gelling agent. Agar extracted from certain sea weeds is a particularly useful gelling agent as it is hydrolysed by only a very few bacteria. It melts at 100 °C but does not solidify until below 40°C.

Growth in liquid media is demonstrated by turbidity. This may be accompanied by a change in colour of an indicator incorporated in the medium to detect acid or alkali production. Gas production resulting from break down of sugars, etc., in the medium may be detected by placing a small inverted test tube (Durham tube) in the main tube to trap some of the gas evolved.

In agar medium the bacteria are trapped on the surface or in the depth of the medium when it is in liquid state. Once the medium solidifies after it cools below 40°C no movement is possible. The cells are only able to grow locally and a cluster of cells, or colony, visible to naked eye is eventually formed.

Liquid media, broths, are dispensed either into rimless test tubes closed by an aluminum cap or a plug of non-absorbent cotton, or into screw capped bottles. Solid, agar media are most commonly poured whilst liquid into shallow, covered dishes (petri dishes or plates) which may contain a small amount of sample.

Media may be selective or non-selective. A nonselective medium permits the growth of all species at the chosen pH, temperature, salinity and oxygen tension. Selective media may contain ingredients which are utilizable only by the selected group and may also contain additional ingredients toxic to others.

Frequently an enrichment medium is used. Such a medium contains a greatly increased amount of a particular ingredient required mainly by the organism to be enriched. The organism outgrows all other types and establishes itself as the dominant organism.

Sterilisation and aseptic techniques

The two most popular methods of laboratory sterilisation (that is, the process of destroying all life forms) both involve heat. Dry heat of 170°C for 90 minutes can be used to sterilise objects which are heat stable such as all glassware. Steam must be used for sterilising aqueous solutions and other items which cannot be placed in an oven. This type of sterilisation is normally carried out in an autoclave at a pressure of 1.06 kg/cm², which corresponds to a temperature of 121°C. The time required for steam sterilisation depends upon the material being sterilised, usually 20 - 30 minutes are sufficient.

Due to the fact that micro-organisms can be present virtually anywhere, it is important to take measures to avoid contamination of microbiological experiments with extraneous bacteria. The measures used to prevent this cross-contamination in microbiological laboratories are collectively known as aseptic technique.

Water samples or bacterial cultures are introduced into sterile media by aseptic procedures to ensure that stray bacteria are not introduced. Asepsis is achieved by:

- Washing one's hands and cleaning the bench area with swab soaked in methylated spirit.
- Working in a dust and draught free area.
- Not touching any part of the container, pipette, etc., which will come in contact with the sample or culture.
- Not removing the lid of a petridish or cap of a test tube longer than absolutely necessary.
- Lightly flaming the top of test tubes, ends of pipettes, necks and stoppers of bottles by a flame before and after adding or withdrawing samples or inocula.
- Transferring culture from one tube to a fresh tube (subculturing) using a wire loop sterilized by heating to redness and cooled.

3. Identification and enumeration

Microscopic examination

It is frequently necessary to look at bacteria under light microscope. Usually an oil immersion lens of X1000 magnification is used with a X10 eyepiece to give a magnification of about X10,000. Before bacteria can be seen under the light microscope they are fixed to a microscope slide by spreading a loop full of cellular mass on a slide and lightly flaming and then stained with a suitable dye.

Gram stain is the most widely used staining method. Those bacteria which after staining with crystal violet resist decolorisation with acetone or alcohol and can not be counter stained are called Gram positive. Those which take up a counter stain such as safranin are called Gram negative. A slide prepared with a mixed culture will show Gram positive organisms blue and Gram negative pink coloured.

The identification of micro-organisms can be accomplished with microscopy. The disadvantage of using a microscope for identification is that it can be time-consuming and require specialist knowledge. Further many different organisms are so similar in morphology that a positive identification cannot be made.

Metabolic reactions

Bacteria can be differentiated on the basis of their metabolic reactions and requirements for nutrition and growth environment. Microbiologists have devised series of tests to identify bacteria even up to the specie level. An organism may be identified on the basis of one or more of the following:

- ability to utilise a particular compound as a substrate for growth
- requirement of an essential compound for growth
- excretion of identified endproducts when using a given substrate
- ability to grow in the presence of a toxicant
- ability to grow in a given environment.

Some of the above tests will be discussed in more detail in the training module on 'Coliforms as indicator of pollution'.

Counts in solid media

Plate count is the most common procedure. The basis of counting is to obtain colonies derived from each cell. These colonies are counted and the count per ml of sample is calculated. Obviously the colonies must be well separated otherwise it would be impossible to count them. A sample containing more than 300 cells per ml must be diluted when a standard 100 mm diameter petri dish is used. The diluent should be a standard salt solution. If the number of cells present is approximately known from previous work, it is not necessary to adhere to the procedure illustrated. For statistically reliable data the plate count should be between 30 and 300. Confidence (95% level) limits for the counts may be calculated from the formulae:

$$\text{Upper limit} = N + 2 (2 + \sqrt{N})$$

$$\text{Lower limit} = N - 2 (1 + \sqrt{N})$$

where N is the number of colonies counted.

The count per ml should be multiplied by the dilution factor.

Counts in liquid media

Counts in liquid media are not as accurate as counts in solid media but they are employed where the organism produces an easily identifiable end product, such as a gas or an acid. The principle employed is dilution to extinction. Broadly stated, if a series of culture tubes are inoculated with equal volumes of a sample then some tubes may show growth while some may not depending upon the concentration of bacteria in the sample and volume of the inoculum. It is then possible to statistically estimate the concentration of the bacteria. In order to increase the statistical reliability of the test a series of tubes are inoculated with varying volumes of the sample or diluted sample. The count thus obtained is called the Most Probable Number (MPN) of cells per stated volume of the sample. The procedure will be

discussed in detail in the modules on 'Coliforms as indicator of pollution' and 'Measurement of coliforms'.

4. Sampling

Samples must be representative of the bulk from which they are taken. Sterilized bottles should be used and aseptic procedures should be followed. Samples from an open body of water should be collected 30 cm below the surface. Samples from water supply systems, which may contain chlorine, should be dechlorinated. Samples should be stored at 4 °C and analyzed within eight hours of collection.

When taking samples care should be taken to ensure that the inside surfaces of the bottle and cap are not touched by the sampler's hand or any other objects as this may lead to contamination of the sample. When the sample is collected, it is important that sufficient airspace (at least 2.5 cm) is left in the bottle to allow mixing by shaking prior to analysis.

5. Good Laboratory Practice

The use of good laboratory practice is an important factor in safeguarding the health and safety of laboratory personnel. It should be remembered that many of the bacteria which are cultured in aquatic microbiological laboratories are capable of producing disease in humans. This, coupled with the fact that, potentially more virulent, pure strains of such bacteria are often being produced, means that there is considerable risk to the health of microbiology laboratory workers if adequate precautions are not taken.

The basis of good practice in a microbiological laboratory can be summed up by the following:

- ensure all necessary equipment and media is sterilised prior to use
- ensure that all sterilised equipment and media is not re-contaminated after sterilisation by allowing it to touch, or rest on, any unsterilised surface
- frequently disinfect hands and working surfaces
- as far as possible, eliminate flies and other insects which can contaminate surfaces, equipment, media and also pass organisms to laboratory personnel
- never pipette by mouth samples which are suspected to have high bacterial concentrations
- wear appropriate protective clothing
- do not eat, drink or smoke in the laboratory
- sterilise contaminated waste materials prior to disposal
- take care to avoid operations which result in bacterial aerosols being formed